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13. ABSTRACT (Maximum 200 Words) This study took several approaches to determine the influence of a single nucleotide polymorphism (SNP) in the matrix metalloproteinase 1 promoter on gene transcription in normal and breast cancer cell lines or tissue. The SNP consists of an extra guanine nucleotide that creates an ETS family transcription factor binding site (2G), and we previously demonstrated that the 2G SNP leads to an increase in transcriptional activity of the MMP-1 promoters containing the 2G SNP can interfere with transcriptional activity of promoters with the 1G SNP, but only at high concentrations of DNA. Analysis of five breast cancer cell lines with transient transfections reveals that the transcriptional effect of the 2G SNP is only observed in one cell line, and this cell line was the only one examined that expressed endogenous MMP-1. The influence of the SNP on endogenous MMP-1 expression was examined in 34 human foreskin fibroblasts (HFFs). From these data we can conclude that the MMP-1 promoter genotype of normal fibroblasts is not predictive of MMP-1 expression. The SNP genotypes of 35 breast cancer patients were determined, and from these data, we can conclude that the SNP has no influence on MMP-1 production from diseased breast tissue. We can block MMP-1 production using short interfering RNA (siRNA) molecules, and have developed a powerful tool to determine the importance of MMP-1 in an in-vivo model of breast cancer metastasis.				
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Introduction:

The lethality of breast cancer is derived from its ability to metastasize, and a critical component of the metastatic mechanism is the degradation of extracellular matrix (ECM). Degradation of ECM is largely mediated by a family of proteins known as matrix metalloproteinases (MMPs). One subfamily of MMPs, the collagenases, is able to degrade stromal collagens type I, II, and III, and the most widely expressed collagenase is collagenase 1 (MMP-1). We have identified a single nucleotide polymorphism (SNP) that enhances the transcriptional activity of the MMP-1 promoter in tumor cell lines and normal stromal cells. The SNP consists of an additional guanine nucleotide creating an Ets transcription factor-binding site (5'-AGGA-3') at position -1607 in the MMP-1 promoter. When this 2G polymorphism is present, transcriptional activity from the MMP-1 promoter, as measured by transient transfections, is increased an average of 6 fold over the 1G polymorphism. A possible role for the SNP in cancer was suggested by observations of increased frequency of the 2G polymorphism in breast cancer cell lines and Japanese cancer patients as compared to normal DNA samples and non-patients(1, 2, 3). The MMP-1 gene is located on chromosome 11 (11q22.2-22.3) in a region associated with loss of heterozygosity in breast cancer patients. The studies outlined in this proposal are designed to characterize the mechanism of enhanced transcription from promoters with the 2G polymorphism, and to determine the effect of the polymorphism on transcription of the endogenous MMP-1 gene. We also hope to elucidate the role of the polymorphism and increased MMP-1 production in tumor invasion using a nude mouse model system. We propose that by understanding mechanisms leading to enhanced production of MMP-1 we can better understand the process of tumor invasion and metastasis, essential elements in the lethality of breast cancer.

Body:**Statement of Work**

Aim/Task 1: *Using a variety of breast cancer cell lines (a) determine the levels of transcription of MMP-1 promoter DNA containing either the 1G or 2G allele, (b) characterize the DNA/protein interactions at these alleles, and (c) examine MMP-1 transcription in adriamycin sensitive vs. adriamycin-resistant breast cancer cells.*

Months1-6: Construct MMP-1 promoter/luciferase constructs containing 1G or 2G linked to either beetle luciferase or sea pansy luciferase, which are detected with different substrates.

These tasks were completed and reported in 2001.

Months 3-9: Begin testing constructs in breast cancer cell lines with transient transfections.

These tasks were completed and reported in 2001. Briefly, the transfections were carried out in A2058 melanoma cells to be consistent with previous transfection experiments. When the A2058 cells were cotransfected with a 1G reporter construct and a 2G reporter construct, promoters with the 2G polymorphism were preferentially transcribed, but only at high concentrations of DNA (figure 1).

Months 9-18: Continue testing cell lines. Compare levels of transcription between adriamycin-sensitive and adriamycin-resistant cell lines. Compare with levels of endogenous MMP-1 expression. Begin analysis of DNA/protein interactions with gel mobility shift assays.

Studies on MMP-1 expression and SNP genotype in breast cancer cell lines were completed and reported in 2001. All six of the breast cancer lines analyzed were homozygous for the 2G SNP, while the normal breast tissue cell line is homozygous for the 1G SNP (table 1). Five of the breast cancer cell lines were analyzed by western blotting (figure 2), and three of the lines were analyzed by northern blotting (figure 3) for MMP-1 production. Only MDA 231 demonstrated high levels of MMP-1. These findings suggest that the presence of the polymorphism is not sufficient for MMP-1 production. Transient transfections revealed that among the five breast cancer lines tested, MDA 231 cells was the only line to demonstrate a significant increase in the transcriptional activity of the 2G SNP promoter over the 1G SNP promoter (14 fold, $p = 0.003$, figure 4). It was concluded that differences in the abilities of these breast cancer cell lines to support the 2G SNP transcriptional enhancement following transient transfections may be due to a lack of appropriate transcription factors.

The experiments performed to look for possible differences in transcription factors were completed and reported in 2002. Electronic mobility shift assays (EMSAs) were run using nuclear extracts from a breast cancer cell line producing high levels of MMP-1 (MDA-231) and a breast cancer cell line producing no detectable MMP-1 (ZR 75). In addition we included nuclear extracts from the MCF 7 adriamycin resistant breast cancer cell line and the A2058 melanoma cell line; both cell lines produce high levels of MMP-1. The nuclear extracts were incubated with radiolabeled DNA probes containing either the 1G or the 2G polymorphism. There was no band characteristic of a MMP-1 producing cell line (figure 5). Additionally, there were no bands specific for DNA probes containing the 2G polymorphism (figure 5). With no differences in the binding patterns, there is no way of identifying proteins that specifically bind to the MMP-1 polymorphism using these EMSA techniques. Last year techniques using real time PCR to determine the mRNA levels of ETS family members were suggested as a possible alternative to EMSA experiments; however those experiments have not commenced.

In 2002 I described a second series of experiments that were completed to address the question of whether the presence of the 2G polymorphism contributes to endogenous MMP-1 gene expression. Initially these experiments were designed for breast cancer cell

lines; however, as detailed above, the breast cancer cell lines were homogeneous in their MMP-1 promoter polymorphism genotype. It was clear that a larger sample size, with more heterogeneity at the MMP-1 promoter, was necessary. I chose human foreskin fibroblasts (HFFs) not only for their availability and probable heterogeneity, but also because stromal cell contributions to metastasis are well recognized and it is possible that the polymorphism influences stromal cell production of MMP-1 during metastasis. The study design included harvesting the fibroblasts from foreskins and genotyping these normal cells for the 1G/2G SNP. Basal MMP-1 production was measured for each cell line. In addition the cells were stimulated with fibroblast growth factor (FGF), epidermal growth factor (EGF), and interleukin 1 (IL-1) to mimic the types of stimuli a stromal cell would see during a metastatic event. Initially I used northern blots to measure the MMP-1 levels; however, a more quantitative analysis was desired. Therefore a real time RT-PCR assay was designed and optimized to measure MMP-1 and GAPDH mRNA levels. To enable quantitative comparisons between PCR assays, standard curves of plasmid DNA were generated with every assay. A manuscript was prepared and submitted to Cancer Research. The manuscript was accepted and published in December of 2002 (4), and a reprint of the article is included in the appendices of this report.

Months 24-36: Finish transfections. Finish characterization of DNA/protein interactions. Analyze data for similarities/dissimilarities. Write PhD thesis.

As detailed above, the transfections were completed, and the results from EMSAs indicate that a new approach to defining which proteins are acting on the site of the polymorphism is needed.

Aim/Task 2: *Using a nude mouse model of human Metastatic breast cancer, and breast cancer lines that are homozygotic and heterozygotic for the 1G/2G/ alleles, (a) trace the development of metastatic tumors relative to their MMP-1 promoter genotype, loss of heterozygosity, location, size, and adriamycin sensitivity phenotype, and (b) examine excised tumors for MMP-1 expression by in-situ hybridization.*

Months 1-12: Begin studies to monitor the development of metastasis in nude mice injected with homozygotic and heterozygotic breast cancer cell lines. Inject 10^6 cells i.v. in tail (10 mice/group). Sacrifice after 4-6 weeks and look for Metastatic lesions in lungs and liver. Excise and size tumors, genotype them, examine for LOH, and begin *in situ* hybridizations for measuring MMP-1 expression.

Months 12-18: Continue with studies, and extend them to determine the earliest time at which a change in genotype may be detected. Sacrifice mice at earlier times and examine inguinal lymph nodes to determine if tumor cells are there and whether they have undergone a change in genotype. Continue *in situ* hybridizations.

Months 18-30: Compare the ability of different breast cancer cell lines (adriamycin-sensitive and adriamycin-resistant) to metastasize in nude mice and correlate with location, size, MMP-1 promoter genotype, and LOH. Finish *in situ* hybridizations.

Months 24-36: Analyze Data. Write PhD thesis.

All of the breast cancer cell lines examined contain only a 2G allele; therefore, LOH studies on these breast cancer lines are not possible. Additionally, an ongoing study in our lab has revealed little to no LOH at the MMP-1 SNP in breast cancer patients, making characterization of breast cancer LOH in mice no longer desirable. The findings of the breast cancer patient study are detailed in the progress report on Department of Defense grant DAMD17-00-1-0221 (principle investigator C.E. Brinckerhoff). Furthermore, during the most recent grant period I performed some work in collaboration with Dr. Dennis Sgroi. He has recently completed a study and published a paper examining gene expression in different disease tissues from a population of breast cancer patients (5). We were interested in examining MMP-1 expression in the varied disease tissues and correlating the stage of disease with MMP-1 expression and MMP-1 polymorphism genotype. Briefly, the study found that there was no statistical correlation between genotype and MMP-1 expression at any stage of the disease. There was; however, an increase in MMP-1 expression over normal tissue in the advanced stages of disease (figure 6). These findings suggest the potential importance of MMP-1 expression in the development of disease. The findings of the study are more completely reported in the annual report for Department of Defense grant DAMD17-00-1-0221 (principle investigator C.E. Brinckerhoff).

As a result of the above findings, the mouse metastasis experiments have been redesigned. While the homogeneity of the MMP-1 promoter polymorphism in the breast cancer cell lines characterized prevents an analysis of LOH in mice, it is possible to use these lines to analyze the influence of MMP-1 expression on the metastatic ability of breast cancer cell lines. As described earlier, MDA 231 cells produce constitutively high levels of MMP-1. Additionally, MDA 231 cells have a well-characterized metastatic potential. I propose stably transfecting MDA 231 cells to block MMP-1 production, and then injecting mice in parallel with MDA 231 cells producing MMP-1, and MMP-1 producing no MMP-1. The location, size, number, and frequency of metastatic events in each group of mice could then be correlated with MMP-1 expression levels in the tumors initially injected. These experiments would address the question of whether MMP-1 is required for metastasis in an *in vivo* system.

I first examined the MMP profile of MDA 231 cells in order to understand which additional MMPs the cell line might be producing. In addition to MMP-1, I developed real time assays to MMP-2 and MMP-9 (the gelatinases) and to MMP-13 (collagenase 3). The partial MMP profile of MDA 231 cells reveals that on plastic the cells produce high levels of MMP-1, no MMP-2 or MMP-9, and very little MMP-13 (table 2). Additional assays to detect MT1-MMP (a membrane bound collagenase) and MMP-8 (collagenase 2) are also in development.

Next I determined whether the MMP-1 phenotype of MDA 231 cells changed when they were exposed to an extracellular matrix. To test this hypothesis, I embedded the MDA-231 cells in a type 1 collagen matrix. I grew them in the matrix for two days,

then harvested the cells in TRIzol, and examined the MMP-1 mRNA expression levels with real time RT-PCR. I found that when compared to a parallel plating of cells on plastic, cells grown in collagen had a significant increase in MMP-1 expression (figure 7). This result demonstrated that the MDA-231 cells would respond to a collagen matrix, and that they would produce more MMP-1 enzyme. I next began to develop the tools to block MMP-1 gene expression in these cells.

As an alternative to antisense technology, I intend to use RNA interference (RNAi) to block MMP-1 expression in MDA-231 cells. RNAi is a relatively new technology that enables the silencing of gene expression in mammalian cells by targeting mRNA molecules with a small RNA oligonucleotide complementary to the mRNA sequence (6). Plasmids capable of producing these short interfering (siRNA) molecules within a target cell were recently developed and are commercially available from Oligoengine (7). My initial experiments were to determine whether the oligonucleotides I designed were capable of blocking MMP-1 gene expression. To avoid complications that may arise from low transfection efficiencies I used a mouse cell line and cotransfected an MMP-1 mRNA expressing plasmid and the RNAi plasmid containing MMP-1 sequence along with a control plasmid expressing enhanced green fluorescent protein. The mouse cells have two advantages as a model. They are readily transfectable, and they do not contain the MMP-1 gene. Therefore the only cells capable of producing MMP-1 message would also be transfected with the RNAi plasmid. The results of these experiments indicate that I can get a significant knockdown of MMP-1 expression using the oligonucleotide I designed (figure 8).

I have recently successfully subcloned the MMP-1 RNAi oligonucleotides into the pSuper Neo GFP vector. This plasmid will stably integrate into the host cell genome, and has the neomycin selectable marker. In addition the siRNA of my design, the vector will produce green fluorescent protein allowing me to identify cells that have been transfected, and to track the cancer cells once they are injected into mice. Future experiments include the creation of two stably transfected cell lines, one producing the MMP-1 specific RNAi molecule, and one producing a scrambled control molecule. After creating these cell lines, I will characterize the MMP profile of the cell lines using real time RT-PCR. I will measure MMP-1, MMP-2, MMP-8, MMP-9, MMP13, and MT1-MMP levels and compare them between cell lines. Then, similar to earlier proposed experiments, I will inject these cell lines in parallel into nude mice. After 4-6 weeks, I will sacrifice the mice, and look for differences in the metastatic profile of each of the stably transfected cell lines. Green fluorescent protein production by the cancer cells will enable me to identify micrometastases more easily. The metastases will be characterized for size, location, number, and MMP profile. These experiments should give me the information to determine whether MMP-1 is playing a role in metastasis of the breast cancer cell line in this in-vivo system.

Key Research Accomplishments:

- Subcloned MMP-1 1G and MMP-1 2G promoter into two different expression vectors
- Cotransfected A2058 melanoma cells with MMP-1 1G and MMP 1 2G reporter vectors

- Observed transcriptional competition at the MMP-1 promoter, but only at high concentrations of DNA
- Genotyped 6 breast cancer cell lines, all 2G homozygotes
- Measured the MMP-1 expression in five breast cancer cell lines by western and three by northern, and noted that only one cell line was producing MMP-1 (MDA 231)
- Performed transient transfections on 5 breast cancer cell lines and noted that the increased transcriptional activity of 2G MMP-1 promoter is only present in 1 of the 5 breast cancer cell lines tested (MDA 231)
- Performed electronic mobility shift experiments with nuclear extracts from breast cancer cell lines that produce MMP-1 and a breast cancer cell line that does not produce MMP-1
- Isolated 60 different HFF lines
- Developed and optimized a MMP-1 real time RT-PCR assay
- Genotyped the MMP-1 promoter of 35 HFF lines
- Measured basal and FGF, EGF, and IL-1 induced MMP-1 production in 32 HFF lines with real time RT-PCR
- Genotyped 35 breast tissue samples from patients with breast cancer
- Developed and optimized real time RT-PCR assays for MMP-2, MMP-9, and MMP-13
- Measured the production of MMP-1, MMP-2, MMP-9, and MMP-13 in a MDA 231 breast cancer cell line
- Developed a siRNA oligonucleotide capable of blocking MMP-1 production in transient transfections
- Subcloned the siRNA oligonucleotide specific for MMP-1 into a stably integrating vector

Reportable outcomes:

An abstract was presented at the 2002 Era of Hope, Department of Defense breast cancer research program meeting.

A paper was published in the December 2002 issue of Cancer Research

Conclusions:

- The data obtained indicate at higher concentrations of DNA promoters containing the 2G polymorphism can interfere with transcriptional activity of promoters containing the 1G polymorphism.
- Transfecting several breast cancer cell lines revealed, the effect of the 2G polymorphism on transcription is only observed in cells expressing endogenous MMP-1.
- The presence of the polymorphism is not sufficient for MMP-1 expression in breast cancer cell lines.
- The MMP-1 promoter genotype was not predictive of basal, FGF induced, EGF induced, or IL-1 induced MMP-1 expression levels in 32 human foreskin

fibroblast (HFF) primary isolates; however, the dispersion of MMP-1 expression levels is greater in a population of cell lines containing the 2G polymorphism.

- The MMP-1 polymorphism genotype is not correlated with MMP-1 production in the disease or normal tissue of breast cancer patients.
- There is an increase in MMP-1 production in disease tissue (ADH, DCIS, IDC) versus normal tissue from breast cancer patients.
- There is an increase in MMP-1 production when MDA 231 cells are exposed to type 1 collagen.
- The lack of a correlation of the polymorphism and MMP-1 production in breast tissue samples suggests that examining the influence of the SNP on metastasis and MMP-1 production in mice may be futile, and would not reflect accurately what is happening in human disease. Likewise, the increase of MMP-1 production in disease tissue over normal tissue from breast cancer patients emphasizes the potential role of MMP-1 in the development of disease. A more appropriate question may be what contribution does MMP-1 production make to metastatic disease. The siRNA tools we have developed may help us answer this question in a mouse model of metastasis. A change to the proposed metastasis protocol in the statement of work would be appropriate to address this new question.

References:

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Appendix 1: Figures and Tables

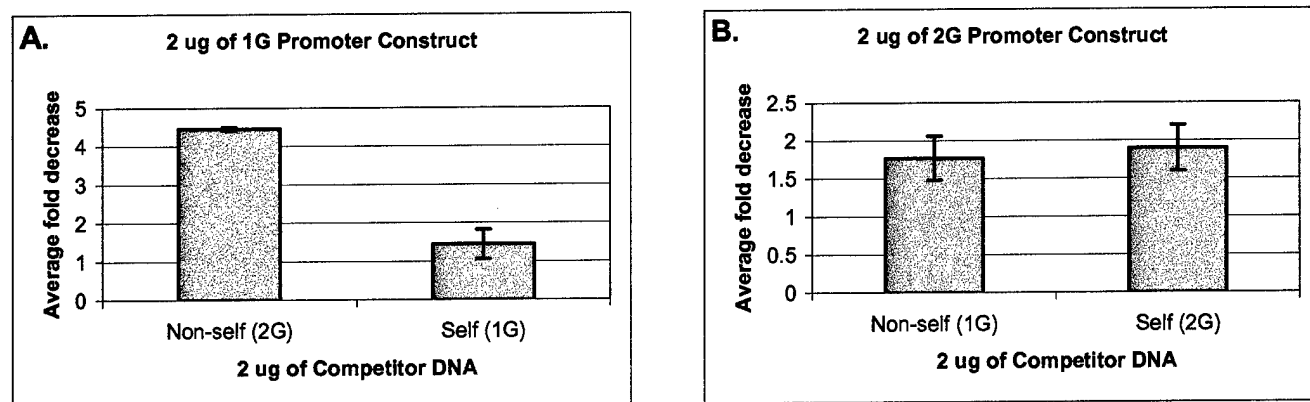


Figure 1. MMP-1 promoters containing the 2G polymorphism can interfere with transcription from promoters containing the 1G polymorphism when cotransfected at higher concentrations of DNA. A, There was a significantly ($p=0.026$) greater decrease in transcriptional activity from $2\mu\text{g}$ of the 1G promoter when an equal amount of 2G competitor was present compared to self-competitor. B, There was no difference in the decrease of transcriptional activity from $2\mu\text{g}$ of 2G promoter when equal amounts of self or non-self competitor were added.

Table 1. Single nucleotide polymorphism (SNP) genotypes of breast cancer cell lines.
*MCF 10A is a normal breast cell line.

Cell Line	SNP Genotype present
SKBr3	2G
T47D	2G
MCF7	2G
MDA 231	2G
ZR75	2G
MDA 468	2G
MCF 10A *	1G

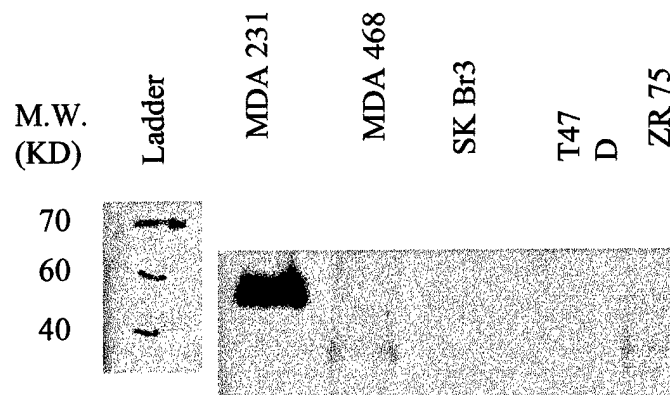


Figure 2. MDA 231 has high levels of MMP-1 protein in serum free culture. Serum free media from 5 breast cancer cell lines were immunoblotted with anti-MMP-1 antibody.

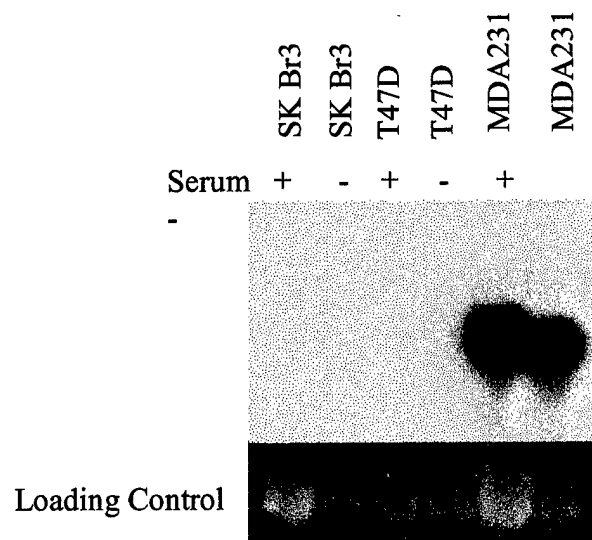
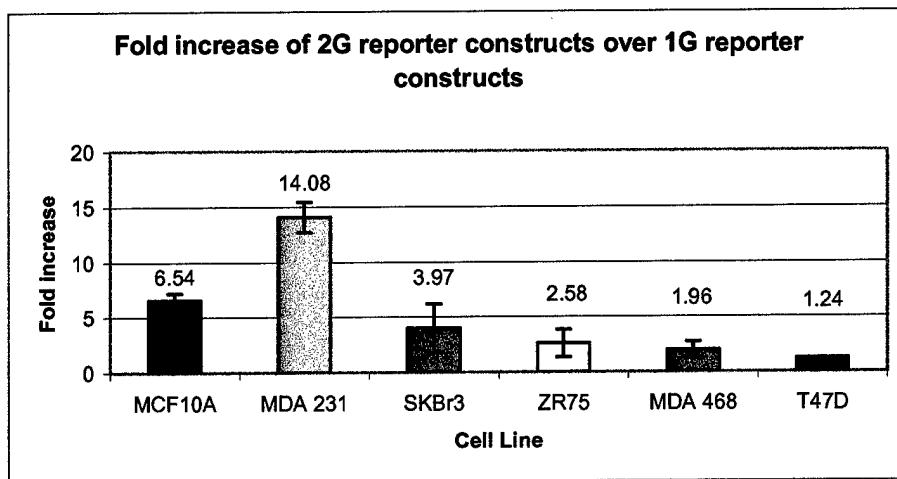


Figure 3. MDA 231 produces high levels of MMP-1 RNA. Northern blots of RNA isolated from three breast cancer cell lines incubated with and without serum revealed that MDA 231 cells produce MMP-1 RNA. The presence of serum does not enhance MMP-1 production.



A. Wyatt

Figure 4. MDA 231 is the only breast cancer cell line that demonstrates a significant increase in transcription when the 2G polymorphism is present. Breast cancer cell lines were transfected with reporter vectors containing the MMP)1 promoter with either the 1G or the 2G SNP. A significant increase in transcription in the presence of the 2G SNP was only seen in the normal breast cell line (MCF 10A, $p=0.013$) or in the MDA 231 breast cancer cell line ($p=0.003$). Paired Student's t-test was performed to determine the significance of the data set for each cell line (SKBr3 $p=0.320$; ZR75 $p=0.292$; MDA 468 and T47D not done).

Cell Line:	<u>Control</u>	<u>MDA 231</u>	<u>ZR 75</u>	<u>A2058</u>	<u>MCF 7_{ADR}</u>
Radiolabeled Probe:	1G 2G	1G 2G	1G 2G	1G 2G	1G 2G

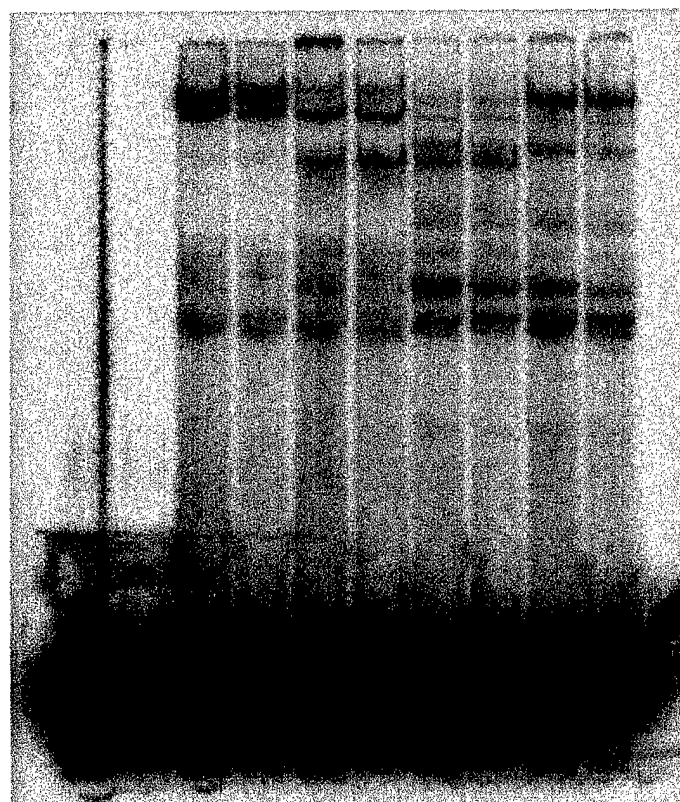


Figure 5. There are no bands specific for MMP-1 producing cells, or for probes containing the 2G polymorphism. Nuclear extracts from Breast cancer cell line that

produce MMP-1 (MDA 231, and MCF 7_{adr}) and nuclear extract from a cell line that does not produce MMP-1 (ZR75) were incubated with radiolabeled probes containing either the 1G or the 2G allele. A2058 nuclear extracts served as a positive control for a cell line producing large amounts of MMP-1. The control lane contained only radiolabeled probe.

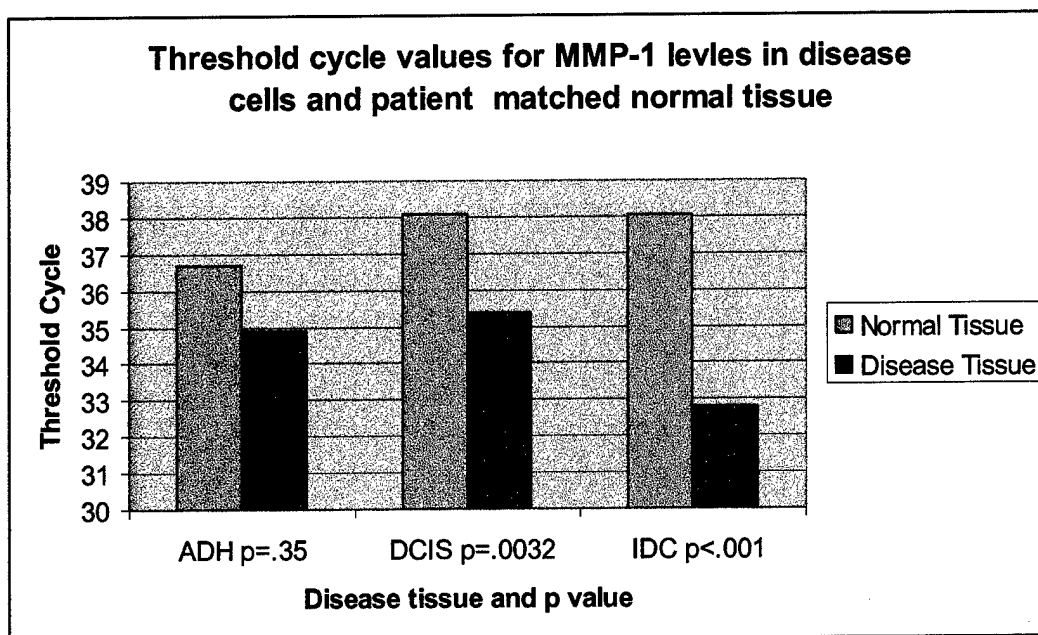
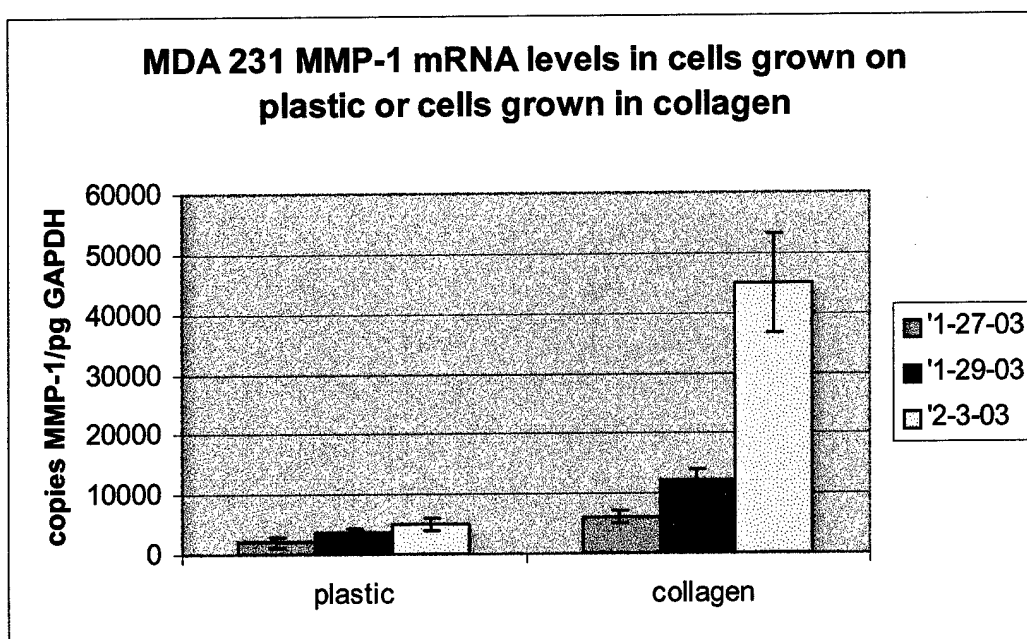


Figure 6. Average Ct values from real time experiments on normal tissue and disease matched tissue. Disease tissue includes Atypical ductal Hyperplasia (ADH), Ductal Carcinoma in situ (DCIS), and Invasive Ductal Carcinoma (IDC) RNA was amplified from laser microdissected disease cells and the MMP-1 levels were quantitated with real time RT PCR. Paired students T tests were run on the values obtained to determine whether there was a significant difference in the Ct for each sample. There is a significant increase in MMP-1 production (a lower Ct) in the DCIS and IDC cells when compared to patient matched normal cells. Details of these studies are more completely reported in the annual report for Department of Defense grant DAMD17-00-1-0221 (principal investigator C.E. Brinckerhoff).

Table 2. Average MMP expression normalized to GAPDH expression. Cells were grown on plastic tissue culture plates, RNA was harvested, and mRNA levels were measured using real time RT-PCR. There is abundant MMP-1 production and little to no MMP-2, MMP-9, or MMP-13

MMP	copies/ pg GAPDH
MMP-1	31,416
MMP-2	0
MMP-9	0
MMP-13	1



Experimental day	P value	Fold increase
1-27-03	0.02	2.9
1-29-03	0.006	3
2-3-03	0.011	9

Figure 7. MMP-1 mRNA levels as determined by real time RT-PCR on three separate experimental days. Cells were grown embedded in a collagen matrix for two days.

MMP-1 levels were normalized to GAPDH expression. P values are reported for a paired t-test used to determine the significance of the increase in MMP-1 expression.

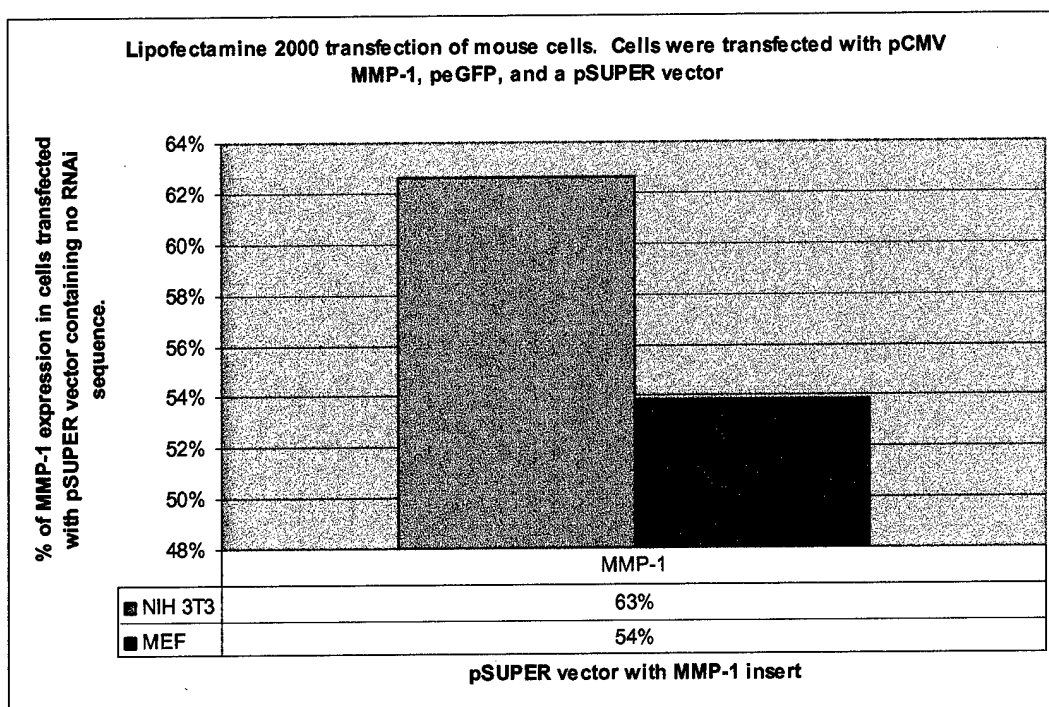


Figure 8. MMP-1 expression levels in two mouse cell lines transfected with pCMV-MMP-1 cDNA, peGFP, and the RNAi pSUPER vector producing either MMP-1 siRNA or no siRNA. MMP-1 levels were normalized to GFP expression levels as determined by real time RT-PCR. Values are expressed as %expression of cells transfected with the empty pSUPER vector.

Appendix 2: C.A. Wyatt, C.I. Coon, J.J. Gibson, C.E. Brinckerhoff, *Cancer Res* **62**. 7200-2. (2002).

Potential for the 2G Single Nucleotide Polymorphism in the Promoter of Matrix Metalloproteinase to Enhance Gene Expression in Normal Stromal Cells¹

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Abstract

The 1G/2G polymorphism of matrix metalloproteinase 1 (MMP-1) affects activity of the promoter in transient transfections, and has been associated with the incidence or invasiveness of five types of cancer. In light of these findings, and because stromal cells may contribute to tumor cell invasion, we used quantitative real-time reverse transcription-PCR to measure endogenous MMP-1 mRNA expression in 34 human foreskin fibroblasts homozygous or heterozygous for the 1G and 2G alleles. We measured basal, cytokine, and growth factor induced MMP-1 mRNA expression. The genotype of the MMP-1 promoter polymorphism was not predictive of mean MMP-1 mRNA expression. However, within the population of cell lines with at least one 2G polymorphism, there were more individuals with higher levels of MMP-1 mRNA after treatment with a cytokine or growth factors. Our data suggest that the presence of the 2G polymorphism does not significantly affect mean expression levels of a population but may increase the potential for an individual to have higher MMP-1 expression in response to growth factors and cytokines.

Introduction

The ECM³ gives structural and functional support to organs and organ systems. In addition to serving as scaffolding for normal biological function, the ECM and its remodeling play major roles in diseases ranging from emphysema to cancer metastasis (1). A family of metalloenzymes, the MMPs, is largely responsible for degradation of the ECM. MMP-1 is one of only a few MMPs capable of degrading interstitial collagen types I, II, and III, and MMP-1 is widely expressed at low levels in normal physiology. However, expression increases markedly in disease pathologies, and increased expression of MMP-1 has been associated with a poor prognosis in several cancers (2).

In 1998, Rutter *et al.* (3) used transient transfections to demonstrate that a SNP in the MMP-1 promoter affected its transcriptional activity. The polymorphism is commonly found in the population and consists of the deletion or insertion (1G or 2G) of a guanine nucleotide at position -1607bp of the MMP-1 promoter. The insertion of the guanine nucleotide produces the consensus sequence for an ETS transcription factor binding site (5'-GGA-3'), and using transient transfections in A2058 melanoma cells, Rutter *et al.* described a

>20-fold increase in transcriptional activity when the 2G polymorphism was present, as well as a 5-10-fold increase in normal fibroblasts. These findings led to the hypothesis that the presence of a 2G polymorphism could increase transcriptional activity of endogenous MMP-1. Because MMP-1 protein levels mirror MMP-1 mRNA expression (4), this increase in transcription may increase the invasive characteristics of cancer cells containing the 2G polymorphism, perhaps through the tumor cells and/or the neighboring stromal cells.

Since that initial study, four separate investigative groups have examined the association between the MMP-1 2G polymorphism and the incidence or invasiveness of five different types of cancer. The 2G polymorphism was positively correlated with an increased risk for developing smoking-associated lung cancer, ovarian cancer, and endometrial carcinoma (5-7). Furthermore, the 2G polymorphism was associated with increased invasiveness of melanoma (8) and colon cancer (9). These epidemiological data establish a potential role for the 2G polymorphism in cancer and metastasis. Theoretically, the 2G polymorphism leads to greater transcriptional activity of the endogenous MMP-1 promoter. Kanamori *et al.* (6) examined MMP-1 mRNA expression in the tumor tissue of ovarian cancer patients using semi-quantitative, radioactive RT-PCR and found higher expression of MMP-1 mRNA in tumor tissues of patients that contained at least one 2G allele in their genome. Likewise, using immunohistochemistry, Nishioka *et al.* (7) found that more patients with the 2G allele had high levels of MMP-1 in their tumor than patients with the 1G allele. These studies begin to deal with the influence of the polymorphism on endogenous MMP-1 expression; however, in the context of metastasis, increased transcriptional activity of the MMP-1 promoter may occur in a variety of cell types including normal stromal cells, tumor-associated fibroblasts, and neoplastic cells.

It is increasingly recognized that stromal cells play a prominent role in facilitating tumor invasion (2, 10-12). Stromal cells respond to growth factors and cytokines in the tumor milieu, such as EGF, bFGF, and IL-1 β , by increasing the production of several MMPs, including MMP-1 (2, 4). In light of the documented associations of the 2G SNP and tumor incidence and invasiveness and because of the important contributions of stromal cells to tumor invasion/metastasis, the present study was designed to determine whether the presence of the 2G polymorphism influences MMP-1 mRNA expression in normal fibroblasts. We used quantitative real-time RT-PCR to measure basal and induced MMP-1 mRNA levels in 34 HFF primary isolates homozygous or heterozygous for the 1G and 2G alleles. We found that the genotype of the MMP-1 promoter polymorphism was not predictive of mean MMP-1 mRNA levels. However, within the population of cells with a 2G allele, there were more individuals with higher levels of MMP-1 mRNA after cytokine or growth factor treatment. Our data suggest that the presence of the 2G polymorphism does not significantly affect mean MMP-1 mRNA expression of a population but may increase the potential for an individual to have higher MMP-1 expression in response to growth factors and cytokines.

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³The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; RT, reverse transcription; EGF, epidermal growth factor; FGF, fibroblast growth factor; bFGF, basic FGF; IL, interleukin; SNP, single nucleotide polymorphism; HFF, human foreskin fibroblast; LH, lactalbumin hydrolysate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CI, confidence interval.

Materials and Methods

Culture of HFFs and Harvesting of RNA. HFFs were isolated and cultured in 50-mm culture dishes in DMEM/20% FCS with penicillin/streptomycin, glutamine, and fungisone as described previously (3). At confluence, cell lines were passed to a 100-mm culture dish, then to five 100-mm culture dishes. When the cell lines reached ~90% confluence, the growth media were removed, and the cells were washed twice with HBSS. The cells were then incubated for 20 h in 5 ml of serum-free media containing 0.2% LH (DMEM), serum-free media plus 10 ng/ml EGF (R&D systems), serum-free media plus 10 ng/ml FGF (Calbiochem), or serum-free media plus 5 ng/ml IL-1 (Promega). RNA was harvested with TRIzol (Promega), and DNA contamination was removed from the RNA samples with DNA-free (Ambion).

Real-Time RT-PCR. RT and real-time PCR were performed using protocols and reagents from Applied Biosystems Taqman RT reagent kit and Sybr Green PCR master mix. Briefly, 2 μ g of DNase treated RNA from a single 100-mm plate were reverse transcribed in a 20- μ l reaction containing 5.5 mM MgCl₂, 500 μ M each dNTP, 2.5 μ M oligo d(T)₁₆, 0.4 units/ μ l RNase inhibitor, and 1.25 units/ μ l Multiscribe reverse transcriptase. The reactions were incubated at 25°C for 5 min, 48°C for 30 min, and then 95°C for 5 min.

Five μ l of each RT reaction were used to amplify MMP-1 mRNA in triplicate real-time PCR reactions and 2 μ l of each RT reaction were used to amplify GAPDH mRNA in duplicate reactions. To enable quantitative comparisons between PCR assays, standard curves were generated with every assay. Serial log dilutions ranging from 1 ng to 100 fg of American Type Culture Collection plasmids pSP6-MMP-1 and pCMV sport6 were used as standards for MMP-1 and GAPDH, respectively. Sequences for the MMP-1 primers were 5'-AGCTAGCTCAGGATGACATTGATG-3' (sense) and 5'-GCCGATGGGCTGGACAG-3' (antisense); and GAPDH primers were 5'-CGACAGTCAGCCGATCTT-3' (sense) and 5'-CCCCATGGTGTCTGAGCG-3' (antisense). The PCRs contained 200 nM each primer and were incubated on a Molecular Dynamics Opticon thermal cycler at 95°C for 10 min, followed by 50 PCR cycles of 95°C for 15 s, and 60°C for 1 min, and a plate read. The PCR cycles were followed by a Sybr green melting curve from 55°C to 90°C. MMP-1 mRNA expression was normalized to GAPDH mRNA expression and is reported as mean copies of MMP-1 message per 500 ng of total RNA \pm the SD.

Genotyping HFFs. DNA was harvested from the 100-mm plates of HFFs using Genra Systems DNA extraction kit. A segment of the MMP-1 promoter containing the 1G/2G polymorphism was amplified by the PCR using 5'-AACCTATTAACCTACCCTTGT-3' as the forward primer and 5'-CCTC-CATTCAAAGATCTTATTAGCATCTCCT-3' as the reverse primer. Each PCR reaction contained 0.2 mM each dNTP, 1.5 mM MgCl₂, 200 nM each primer, 1 unit platinum TaqDNA polymerase (Invitrogen), and 1 \times PCR buffer (Invitrogen). The reactions were subjected to a hot start of 94°C for 2 min followed by 35 cycles of 94°C for 30s, 55°C for 30 s, and 72°C for 1 min. The PCR product was purified using a Qiagen PCR purification kit, and then the product was sequenced using a nested primer (5'-AGTGT-TCTTTGGTCTCTGC-3') and the Applied Biosystems dig dye terminator sequencing kit.

Table 1 Mean, SD, median, and upper quartiles of HFF MMP-1 expression levels in copies of MMP-1 mRNA/500 ng total RNA

Treatment	Genotype	n	Mean	SD	Median	Upper quartile
LH	G	9	143.98	290.36	28.77	96.66
	GG	5	377.08	725.92	23.96	181.42
	H	15	156.90	233.03	39.34	138.41
EGF	G	9	1228.19	2393.17	125.75	895.25
	GG	8	3308.71	5730.64	268.99	5802.92
	H	17	3476.73	9707.42	301.71	2382.90
IL-1	G	9	2882.73	7281.39	109.53	897.65
	GG	6	7785.92	12198.79	2642.90	9896.99
	H	17	4955.16	10892.82	589.88	2736.04
FGF	G	9	11826.66	31257.92	1232.33	1584.80
	GG	7	8848.03	13825.75	2016.82	14732.55
	H	17	10470.57	25943.70	1546.42	6052.52

Table 2 Fold increase of MMP-1 expression after treatments

Fold increase and Ps are calculated from the log transformed model-based estimates of mean expression.

Genotype	Treatment	Fold increase over LH	95% CI	P
G	EGF	6.83	1.34-34.88	0.0214
	IL-1	10.06	1.97-51.34	0.0060
	FGF	39.83	7.80-203.28	<0.0001
GG	EGF	17.46	2.28-133.84	0.0065
	IL-1	37.68	4.33-328.15	0.0013
	FGF	83.71	10.62-660.13	<0.0001
H	EGF	10.14	2.95-34.83	0.0003
	IL-1	15.55	4.53-53.39	<0.0001
	FGF	39.19	11.41-134.62	<0.0001

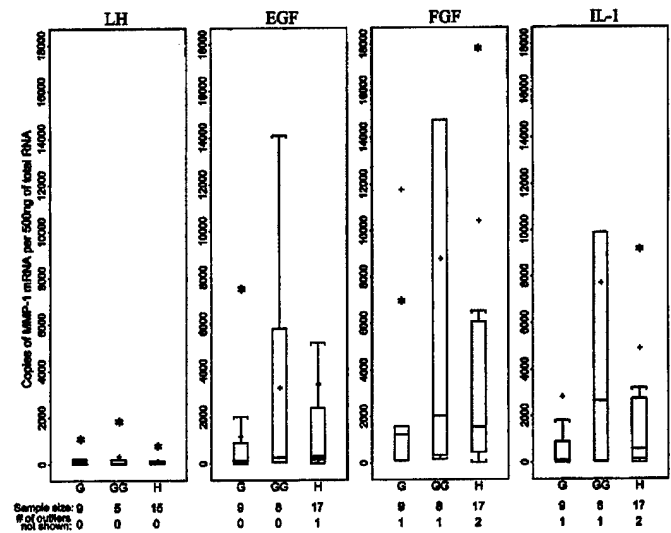


Fig. 1. Box plots of the distribution of MMP-1 expression levels in 1G homozygous (G), 2G homozygous (GG), or heterozygous (H) cell populations incubated in serum free DMEM containing 0.2% LH, 10 ng/ml EGF, 10 ng/ml FGF, or 5 ng/ml IL-1. Each box represents the interquartile range (25th-75th percentile) of the population. The line within each box, the median value. +, the mean of each population. Data points lying beyond the upper fences (vertical lines above the boxes) are considered outliers (*). (See "Methods" for an explanation of this calculation.) The number beneath each plot, the number of outliers beyond the scale of the plots.

Statistical Methods. Random coefficient regression was used to model the relationship between MMP-1 expression and the cell genotype and treatment applied. Log transformation was applied to the MMP-1 expression values for variance stabilization. 95% CIs are given for all model-based estimates. The formula used for calculating the upper fence was the upper quartile + 1.5 * (upper quartile - lower quartile). The formula for calculating the lower fence was the lower quartile - 1.5 * (upper quartile - lower quartile).

Results and Discussion

The HFFs were primary isolates from normal tissue; therefore, it was assumed that they were diploid cell lines. Distribution of genotypes in the 34 HFF cell lines isolated agrees with our previous findings (3). Approximately 25% of the cells were homozygous for the 1G polymorphism, ~25% were homozygous for the 2G allele, and ~50% were heterozygous (Table 1). Also in keeping with our previous report (3), expression of MMP-1 mRNA in unstimulated HFF cell lines was low. Analysis of MMP-1 expression in HFFs by real time RT-PCR revealed the average basal expression for 1G homozygotes to be 144 ± 290 (mean \pm SD) copies of MMP-1 message/500 ng RNA, 2G homozygotes averaged 377 ± 726 copies of MMP-1 message/500 ng RNA, and heterozygotes produced an average of

nearly 157 ± 233 copies of MMP-1 message/500 ng RNA (Table 1). Although these findings are suggestive of a biological trend in which the average basal expression is higher in cells homozygous for the 2G allele, variations in the level of expression among the samples precluded statistical significance ($P > 0.05$).

HFFs treated with EGF, IL-1, or FGF had statistically significant increases in mean MMP-1 expression, confirming earlier published results (Table 2; Refs. 2, 13). A comparison of MMP-1 expression among genotypes revealed that mean MMP-1 expression in EGF-stimulated 2G homozygous cells was greater than the corresponding expression in 1G homozygous cells ($3,309 \pm 5,700$ copies of MMP-1 mRNA/500 ng total RNA and $1,228 \pm 2,393$ copies of MMP-1 mRNA/500 ng total RNA, respectively; Table 1). Likewise, mean MMP-1 expression in IL-1-stimulated HFFs was greater for the 2G homozygous population than for the 1G homozygous population ($7,786 \pm 12,199$ copies of MMP-1 mRNA/500 ng total RNA and $2,883 \pm 7,281$ copies of MMP-1 mRNA/500 ng total RNA, respectively). However, after bFGF treatment, homozygous 2G cells had lower average MMP-1 expression ($8,848 \pm 13,826$ copies of MMP-1 mRNA/500 ng total RNA) than did 1G homozygous cells ($11,827 \pm 31,257$ copies of MMP-1 mRNA/500 ng total RNA). Similar to basal MMP-1 expression, stimulated MMP-1 expression varied widely among cell lines, and none of these differences in expression were statistically significant with respect to the 1G versus 2G allele ($P > 0.05$). Similar variability in MMP-1 production that was related to the 1G/2G polymorphism was recently described in amnion cells from patients with preterm premature rupture of fetal membranes (14). This variability in SNP expression is commonly seen and has been attributed to environmental and physiological factors that complicate the analysis of genetic differences from individual to individual (15).

Although the presence of a 2G MMP-1 promoter does not significantly affect mean MMP-1 expression levels, it does influence the dispersion of MMP-1 expression levels within a population of cell lines (Table 1; Fig. 1). The upper quartiles of MMP-1 expression in HFFs clearly demonstrate this. Heterozygous EGF-, bFGF-, and IL-1-treated HFFs had upper quartiles that were 2.5, 4, and 3 times greater than their respective quartiles in 1G homozygous HFF cells. The presence of an additional 2G allele (the 2G homozygotes) increases the extremes of MMP-1 expression further. In the EGF-, bFGF-, and IL-1 treated 2G homozygous cell populations, the upper quartiles were 6.5, 9.3, and 11 times greater than their respective quartiles in 1G homozygous cells. The presence of more individual cell lines with higher MMP-1 mRNA expression in the populations containing a 2G allele suggests that, although the MMP-1 promoter genotype is not predictive of the mean MMP-1 expression level of a population, there may be a greater potential for a given individual with a 2G polymorphism to have higher MMP-1 expression.

A positive correlation has been established between the presence of the 2G polymorphism and the incidence or invasiveness of five different cancers (5–9). Additionally, two of these studies demonstrated higher MMP-1 expression in tumor tissues containing the 2G polymorphism compared with tumor tissues that contained only the 1G polymorphism (6, 7). The differences between findings in studies on cancer and our findings may be explained by the differences in the cell types being examined. The HFF lines described here represent an unselected population of normal cells derived from individuals and tissues not involved with disease. In contrast, Kanamori *et al.* and Nishioka *et al.* measured the association of MMP-1 expression and the 2G allele in tumor tissues from a population selected for the presence of disease (6, 7). It is possible the perturbation of transcription in neoplastic cells, such as those examined by Kanamori *et al.* and

Nishioka *et al.*, potentiates the observed influence of the 2G polymorphism on high levels of MMP-1 expression. This argument implies that the MMP-1 polymorphism affects only neoplastic cell MMP-1 expression. However, there is some evidence suggesting that stromal cells associated with neoplastic cells are different from stromal cells in other regions of the body (12), and therefore, our findings in foreskin fibroblasts might not be predictive of the findings of a similar study in tumor-associated fibroblasts (16). Additionally, a potential role for the MMP-1 polymorphism in a nonneoplastic disease was recently established by Fujimoto *et al.* (14) when they found that there is a modest but significant increased risk for the development of preterm premature rupture of fetal membranes in African-American women with a 2G polymorphism in their MMP-1 promoter. Future, quantitative examinations of the influence of the 2G MMP-1 promoter genotype and MMP-1 expression in neoplastic cells and tumor-associated fibroblasts may further clarify how the 2G polymorphism is involved in the development and invasiveness of cancer.

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